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ORIGINAL PAPER

Fluctuations in Cytosolic Calcium Regulate the Neuronal Malate–Aspartate NADH Shuttle: Implications for Neuronal Energy Metabolism

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Abstract The malate–aspartate NADH shuttle (MAS) operates in neurons and other cells to translocate reducing equivalents from the cytosol to the mitochondrial matrix, thus allowing a continued flux through the glycolytic pathway and metabolism of extracellular lactate. Recent discoveries have taught us that MAS is regulated by fluctuations in cytosolic Ca^{2+} levels, and that this regulation is required to maintain a tight coupling between neuronal activity and mitochondrial respiration and oxidative phosphorylation. At cytosolic Ca^{2+} fluctuations below the threshold of the mitochondrial calcium uniporter, there is a positive correlation between Ca^{2+} and MAS activity; however, if cytosolic Ca^{2+} increases above the threshold, MAS activity is thought to be reduced by an intricate mechanism. The latter forces the neurons to partly rely on anaerobic glycolysis producing lactate that may be metabolized subsequently, by neurons or other cells. In this review, we will discuss the evidence for Ca^{2+}-mediated regulation of MAS that have been uncovered over the last decade or so, together with the need for further verification, and examine the metabolic ramifications for neurons.

Keywords Mitochondria · Malate–aspartate NADH shuttle · Calcium · Neuron · Metabolism

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Introduction

The malate–aspartate NADH shuttle (MAS) is the main pathway whereby reducing equivalents from cytosolic NADH are transferred to mitochondria. It is composed of two sets of enzymes, malate dehydrogenase and aspartate aminotransferase each with mitochondrial and cytosolic localization, and two mitochondrial carriers, that of oxoglutarate/malate, OGC/Slc25a11 and those of aspartate/glutamate, the AGCs. The AGCs have two isoforms in mammals, AGC1/aralar/Slc25a12 and AGC2/citrin/Slc25a13 [1], and AGC1 is the main brain isoform [2–4] with only marginal expression of AGC2 in a few brain nuclei [5]. The AGCs belong to a family of Ca^{2+} activated mitochondrial carriers which stand out because of the presence of EF-hand Ca^{2+}-binding motifs on the N-terminal half of the protein. These motifs face the intermembrane space and can sense extramitochondrial Ca^{2+} [2, 6, 7]. Most of the glucose utilized by brain neurons is oxidized in mitochondria, and this requires a substantial transfer of reducing equivalents from cytosolic NADH to mitochondria through MAS [8]. In vivo, AGC1 may largely be present in neurons [9] although both mRNA and functional protein has been detected in freshly isolated mouse astrocytes [10], and aralar was identified as an interaction partner to the astrocytic glutamate transport,

GLT-1 [11]. In this review, we will focus on the role of Ca^{2+} activation of AGC1-MAS in response to neuronal workloads under conditions of varying intensity stimulation.

Malate–Aspartate Shuttle in Isolated Brain Mitochondria and Intact Neurons: Activation by Fluctuations in Cytosolic Ca^{2+}

To study the regulation of MAS, Contreras and coworkers reconstituted MAS in isolated brain mitochondria and studied the kinetics of activation by Ca^{2+} (see references below). MAS is active in a Ca^{2+}-free medium but its activity is increased about three-fold in the presence of extramitochondrial Ca^{2+}, with half maximal activation at 300 nM Ca^{2+}. This involves a change in Vmax, but not the affinity for glutamate [12, 13]. These experiments were carried out in the presence of ruthenium red, an inhibitor of the mitochondrial Ca^{2+} uniporter clearly identifying extramitochondrial rather than matrix Ca^{2+} as the cause of activation. MAS activity feeds reducing equivalents from cytosolic NADH to the matrix and mitochondrial oxygen consumption fueled by glutamate and malate depends on MAS activity [14]; therefore, extramitochondrial Ca^{2+} activation of MAS is expected to increase respiration on these substrates in isolated mitochondria in the presence of high ADP levels. Indeed, Gellerich and coworkers using high resolution respirometry found exactly that to be the case, with a Kd for Ca^{2+} activation of respiration of about 300 nM [15]. Ca^{2+} activation was independent of the presence of ruthenium red, consistent with a role of extramitochondrial rather than matrix Ca^{2+} on activation [15]. Further work of Gellerich and coworkers suggested that the main function of MAS activation by Ca^{2+} in regulating respiration was the control of pyruvate supply [16]. This conclusion was inferred from experiments of MAS reconstitution with brain mitochondria in the presence of lactate and lactate dehydrogenase (LDH). In this system, Ca^{2+} activation of MAS acted upon the NADH/NAD^+ couple in the extramitochondrial medium to divert lactate into pyruvate, which then fed mitochondria. In this case, a large part of the Ca^{2+}-dependent increase in respiration was blocked by cinnamate, an inhibitor of pyruvate transport in mitochondria [16]. Therefore, the reducing equivalent transfer through MAS would add to pyruvate supply (also dependent on MAS) to fully increase respiration under these conditions [16]. On the whole, Ca^{2+}-activation of MAS through aralar/AGC1 would provide the neurons (or any cell) with the capacity to increase respiration on pyruvate if needed, acting as a gas pedal [16, 17].

However, evidence that these mechanisms really operate in intact neurons with the actual concentrations of substrates was lacking. On the one hand, even with glucose as the main energy source, neuronal mitochondria may face a variety of respiratory substrates [8]. On the other, the role of Ca^{2+} itself as a regulator of neuronal respiration has been challenged by the finding of a lack of response to Ca^{2+} in oxygen consumption of cerebellar neurons [18]. These issues have been recently addressed in cerebrocortical neurons using physiological glucose concentrations [19]. Calcium was clearly shown to play a role in regulating workload-stimulated neuronal respiration. Interestingly, MAS played a very important role in this stimulation. The lack of aralar/AGC1 completely suppressed the Ca^{2+}-dependent responses to the small-to-moderate workloads caused by carbachol, which mobilizes endoplasmic reticulum (ER) Ca^{2+}, and K^+-depolarization, which allows Ca^{2+} entry via voltage-gated Ca^{2+} channels. In both cases the response was restored by the addition of pyruvate, clearly showing a major role of MAS in providing pyruvate via glycolysis to neuronal mitochondria. In fact, even under conditions in which Ca^{2+} was taken up into mitochondria resulting in increased matrix NADH levels [20] and pyruvate dehydrogenase activation [21], neuronal respiration was not stimulated, indicating that the role played by MAS in increasing pyruvate supply to mitochondria is required to fuel respiration on glucose [22]. MAS activity was also required to respond to the high workload imposed by veratridine, which opens voltage-gated Na^+ channels; in its absence, the large Ca^{2+}-dependent increase in respiration was reduced by 70 %, but the residual 30 % increase is most likely due to matrix Ca^{2+} effects caused after Ca^{2+} entry in mitochondria [19].

Malate Aspartate Shuttle in Isolated Brain Mitochondria and Intact Neurons: Inhibition by Matrix Ca^{2+}

Regulation by Ca^{2+} of reconstituted MAS in brain mitochondria differed if ruthenium red was not included in the assays [12]. In that case, low Ca^{2+} concentrations still activated MAS, but at higher Ca^{2+} concentrations in the micromolar range MAS activity decreased [12]. This paradoxical result was not due to mitochondria dysfunction due to the opening of the permeability transition pore caused by high matrix Ca^{2+}, as it was not modified in the presence of cyclosporin. Rather, it was traced to effects of matrix Ca^{2+} on the second mitochondrial carrier of MAS, the malate oxoglutarate carrier, OGC. Indeed, reconstitution of the OGC in brain mitochondria exposed to different Ca^{2+} loads in the absence of ruthenium red indicated that the OGC export of α-ketoglutarate (α-KG) decreased with the Ca^{2+} taken up in mitochondria [12]. Interestingly, this effect was reversible, and the activity of the carrier was

restored after allowing Ca^{2+} efflux from mitochondria via the Na^{+}/Ca^{2+} exchanger [12]. The effect of matrix Ca^{2+} on the OGC was attributed to matrix Ca^{2+} activation of α-ketoglutarate dehydrogenase (α-KGDH), and consequent inhibition of OGC, as suggested for heart mitochondria [23, 24]. Both activities have similar Km value for α-KG (1.5 and 2.1 mM for the OGC and α-KGDH, respectively [25, 26]). Upon entry through the mitochondrial uniporter, Ca^{2+} stimulates α-KGDH by reducing its Km value for α-KG (from 2.1 to 0.16 mM, with an $S_{0.5}$ of around 1.2 μM for calcium [25]). In this situation, OGC will be at a disadvantage, and thus α-KG efflux will become impaired (see Fig. 4, Ref. [7]). This competition of the two pathways for a common substrate is consistent with the findings of O'Donnell et al. [23], who reported a fourfold reduction of MAS activity in heart under high workload conditions. However, it must be kept in mind that the experiments on MAS inhibition by matrix Ca^{2+} in isolated mitochondria were carried out in the absence of pyruvate (or lactate as pyruvate donor). As indicated by Gellerich [16] in isolated mitochondria and by Llorente-Folch et al. [19, 21] and Rueda et al. [22] for intact neurons, the main function of MAS activation is to stimulate pyruvate uptake in mitochondria, and it is possible that the extra supply of pyruvate caused by Ca^{2+}-activation of MAS prevents a fall in α-KG levels and inhibition of the OGC. In fact, Gellerich et al. [16] did not detect any inhibitory effect of Ca^{2+} on respiration on malate + glutamate within the 10 nM to 1 μM range in the presence of lactate as a pyruvate donor. Therefore, in isolated brain mitochondria inhibition of MAS by matrix Ca^{2+} caused by Ca^{2+} concentrations up to 1 μM probably depends upon the absence of pyruvate supply. However, glucose-derived pyruvate supply may become limiting in intact neurons under conditions of intense workloads and large Ca^{2+} signals [27]. Therefore, the extent to which the inhibition of OGC by matrix Ca^{2+} occurs in vivo remains an open question.

The bulk of ATP production in neurons, and indeed most eukaryotic cells, is via oxidative phosphorylation in the mitochondria, with glycolysis providing only a marginal amount of ATP [8]. In neurons, the pyruvate fueling mitochondrial oxidative metabolism may be derived from either glucose or lactate present in the extracellular space [8]. Reversible inhibition of the MAS by intermittent activation of the mitochondrial Ca^{2+} uniporter and thus fluctuating matrix Ca^{2+} levels during neurotransmission activity, as shown by Contreras et al. [12], will have a direct effect on the neuron's ability to process glucose and lactate since the production of pyruvate from both glucose and lactate requires a continuous oxidation of cytosolic NADH generated in glycolysis and the LDH-catalyzed reaction, respectively [8]. Thus, what we propose is that during peak neurotransmission in which the (post-synaptic) cytosolic Ca^{2+} levels rise sufficiently high to surpass the mitochondrial Ca^{2+} uniporter's threshold for Ca^{2+} accumulation in the mitochondrial matrix, MAS becomes inhibited due to a Ca^{2+}-induced increase in the affinity of α-KGDH for the substrate, α-KG, thus competing with the mitochondrial OGC, a key component of the MAS as discussed above [12, 28]. It is important to note, that in situ the MAS is not likely to be completely but only partially inhibited; in other words, when this mechanism is manifest, it will limit the mitochondria's ability to re-oxidize cytosolic NADH, not completely abolish it.

We have previously independently suggested that this intermittent, reversible inhibition of the MAS leads to the neurons not being able to employ lactate as a substrate during peaks in mitochondrial matrix Ca^{2+} [12, 28, 29]; on the contrary, they may actually become net producers of lactate contributing to the rise in extracellular lactate observed during neurotransmission activity [30]. The latter is corroborated by an in vivo study in rodent cerebellum showing that inhibition of post-synaptic glutamate receptors abolished increases in extracellular lactate levels induced via climbing fibre stimulation of cerebellar Purkinje cells [31]. We have previously shown that active cultured neurons releases lactate to the extracellular medium [29] and that glucose, but not lactate metabolism is up-regulated during neurotransmission activity [28, 32].

This view is not consistent with the 'classical' astrocyte-neuron lactate shuttle hypothesis, stating that lactate flows in only one direction, i.e. from astrocytes to neurons [33]. It is, however, consistent with a more nuanced view of lactate dynamics where extracellular lactate originates from, and is metabolized by, multiple cell types, where lactate is scattered from the activated brain area via the astrocytic syncytium [34], and where lactate plays roles other than that of an energy substrate, e.g. redox signaling to switch on neuronal plasticity genes [35]. It still seem appropriate to challenge the conservative and somewhat static view on lactate shuttling between neurons and astrocytes that almost seem to predetermine conclusions from new studies regarding metabolite fluxes, as exemplified by a recent study on the interaction between oligodendrocytes and the axonal compartment of neurons [36, 37]. One issue that remains unsettled in this latter study is the fact that axons metabolizing lactate (or pyruvate) only are not able to operate the pentose phosphate shuttle. Thus, these long neuronal white matter projections cannot synthesize nucleotides and re-oxidize NADPH locally.

However, not all neuronal mitochondria may be affected by Ca^{2+}-induced inhibition of MAS. We know that mitochondria are heterogeneous in terms of their proteome and function among different organs, within organs, and likely within the same cell [38–41]. Is it likely that mitochondria

within the same neuron are different, or that they experience differences in their cellular environment that causes them to respond distinctly to different stimuli? We know from metabolic labeling experiments that more than one cellular pool of a given metabolite may exist, showing differential turn over rates or distinct access to label other metabolites [42]; these are very likely linked to heterogeneity of mitochondria. Mitochondria are very dynamic organelles in terms of changing their morphology and location within the cell, and it has been shown that moving neuronal mitochondria pauses near sites of high cytosolic Ca^{2+} levels [43] suggesting that mitochondrial heterogeneity in terms of metabolism may arise due to proximity to Ca^{2+} microdomains. Based on experiments in cultured neurons, it was suggested that only a subset of the mitochondrial pool in neurons respond to elevations in cytosolic Ca^{2+} levels, and that those mitochondria that do respond, primarily metabolize glucose-derived pyruvate as compared to that derived from oxidation of lactate by LDH [29]. Thus, when Ca^{2+}-induced inhibition of MAS is manifest in this subset of mitochondria during neurotransmission activity, the neurons will produce lactate from glucose and release it to the extracellular environment [29].

Concluding Remarks and Future Directions

As repeatedly pointed out by Gerry Dienel, brain activation involves increased lactate production [44–47]. As mentioned above, a well-known proposal is that during brain activation, lactate is mainly produced by astrocytes, and consumed in neurons [33, 48]. However, other studies have concluded that neurons still consume glucose during brain activation, and that the excess of lactate produced is probably removed from the brain through the circulation [45, 46, 49, 50]. The results of Bak et al. [28, 29] show that neurons may be one of the sites of lactate production during activation, perhaps due to intermittent MAS inhibition under high activating conditions, consistent with the role of matrix Ca^{2+} on regulation of OGC and MAS [12].

Admittedly, much work needs to be done to clarify the possible role of OGC inhibition by matrix Ca^{2+} in vivo during brain activation. This has not been experimentally addressed so far. In addition, some of the earlier work on overloaded heart, which indicated an inhibition of the OGC with increasing workload, needs further verification. For example, the original finding of a reduced OGC mediated outflow of α-KG from mitochondria in the dobutamine—stimulated heart from mongrel dogs was obtained after metabolic labeling with [2-^{13}C]-acetate [23]. However, in a second study by the same group carried out in rat heart subject to pressure overload after metabolic labeling with [2,4-^{13}C]-butyrate, OGC activity increased with workload, with no signs of inhibition [51]. This is also consistent with the maintenance or increase in glutamate labeling from [2-^{13}C]pyruvate in heart of dobutamine-treated rats [52]. Therefore, it is likely that the choice of animal model, labeling substrate or workload is influencing the results obtained.

Another concern to be added refers to the changes in metabolite levels that may be predicted if OGC activity is inhibited. Under normal, basal conditions MAS is regulated at the level of its only irreversible step in polarized mitochondria, glutamate + H^+/aspartate exchange catalyzed by aralar/AGC1. This step has the lowest V_{max} of the MAS components [12], is driven by the proton electrochemical gradient, and is the point of regulation by Ca^{2+}. Changes in the activity of the AGC are expected to influence the distribution of glutamate/aspartate between mitochondria and cytosol. A decrease in AGC1 activity would increase cytosolic glutamate but decrease cytosolic aspartate, probably the major pools of these amino acids, suggesting that an increase in the glutamate to aspartate ratio may be an indicator of low AGC and MAS activity. This is the situation in neurons lacking AGC1 [9, 14], and also that observed in different stimulation conditions both in vitro and in vivo ([12]; Table 3). However, the inhibition of MAS by matrix Ca^{2+} is not due to changes in AGC activity, but to a decrease in OGC activity, which may now become limiting for MAS [12]. If this gives rise to a decrease in cytosolic α-KG, it may affect the much larger cytosolic glutamate pool, but that remains to be established. Thus, much work still needs to be done to clarify the intricate details of Ca^{2+}-mediated regulation of MAS in neurons, and to verify the findings in vivo.

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